USER GUIDE

Ovation® SP+ Ultralow Library Systems

PART NOS. 8133, 8134

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I. Introduction

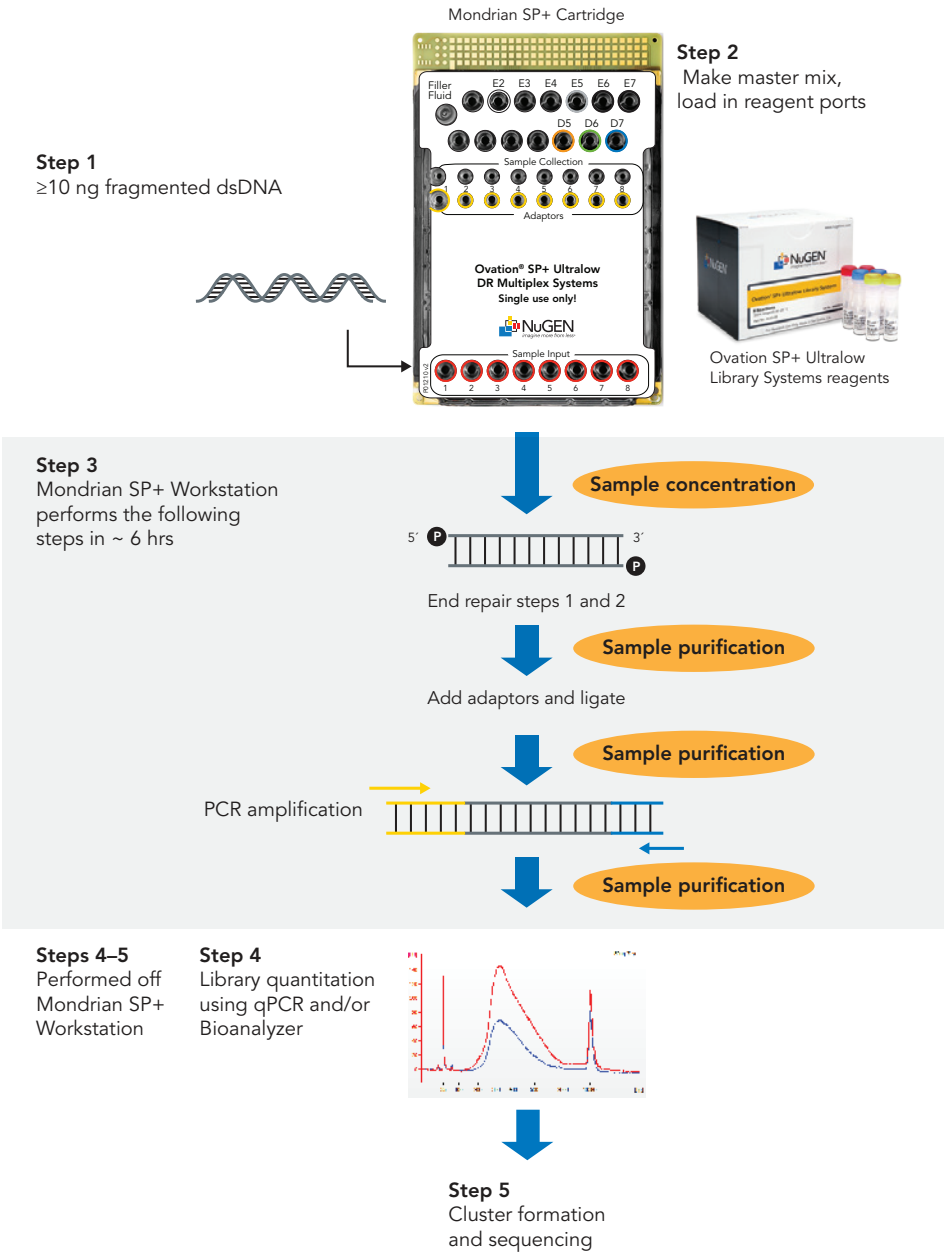
A. Background

The Ovation® SP+ Ultralow DR Multiplex Systems 1–8 and 9–16 (Part Nos. 8133 and 8134) are complete reagent, cartridge and protocol packages for the simple automation of DNA library preparation protocols on the Mondrian SP+ Workstation. These systems enable library preparation for next generation sequencing starting with as little as 10 ng of sheared, double-stranded DNA (dsDNA). The resulting libraries are suitable for a wide range of sequencing applications including RNA-Seq, genomic sequencing, amplicon sequencing, ChIP-Seq and more. As shown in Figure 1, the streamlined workflow consists of five steps:

1. Fragmentation of either genomic DNA or double-stranded cDNA to produce the assay template
2. Addition of template and reagents to the Mondrian SP+ Cartridge
3. Hands-free automation of the following assay steps on the Mondrian SP+ Workstation:
   - Sample concentration
   - End repair
   - Sample purification
   - Adaptor ligation
   - Library amplification
   - Amplified library purification
4. Quantitation of the purified, amplified library
5. Cluster formation and sequencing

I. Introduction

Figure 1. The Ovation SP+ Ultralow Library Systems workflow.



The entire workflow requires approximately 45 minutes of hands-on time to prepare and load reagent master mixes onto the cartridge and retrieve purified, amplified libraries. No manual bead or gel purification steps are required. Starting with as little

as 10 ng of fragmented dsDNA, the protocol takes approximately 7 hours to complete, depending on the number of library amplification cycles performed. The final libraries are ready for cluster formation and single read or paired-end sequencing.

In addition to genomic and other double-stranded DNA sources, the Ovation SP+ Ultralow Library Systems have been designed for seamless integration with NuGEN's Ovation WGA FFPE System (Part No. 6200), Ovation RNA-Seq System V2 (Part No. 7102), Ovation RNA-Seq FFPE System (Part No. 7150), Ovation 3'-DGE System (Part No. 7200), Ovation Prokaryotic RNA-Seq System (Part No. 9030) and Encore Target Capture Module (Part No. 0332) to enable a complete end-to-end solution for transcriptome library construction starting with total RNA.

The Ovation SP+ Ultralow DR Multiplex Systems 1–8 (Part No. 8133) and 9–16 (Part No. 8134) each provide eight unique dedicated read barcoded adaptors to prepare libraries for multiplex sequencing. Together, these two kits enable up to 16-plex sequencing, although multiplexing is not required in order to use this kit.

Important: Please familiarize yourself with the Mondrian SP+ Cartridge Handling instructions in Appendix B and the Mondrian SP+ Workstation User Manual (M01322) prior to beginning work with the Ovation SP+ Ultralow Library Systems.

B. Performance Specifications

The Ovation SP+ Ultralow Library Systems are designed to produce DNA libraries suitable for either single read or paired-end sequencing on the Illumina Genome Analyzer IIx/IIe (GAII), MiSeq™, HiScan™ SQ or HiSeq™ NGS platforms without gel-based size selection, using 10–100 ng input of dsDNA. The Ovation SP+ Ultralow Library Systems generate libraries ready for quantitation in approximately 7 hours.

C. Quality Control

Every lot of the Ovation SP+ Ultralow DR Multiplex Systems 1–8 and 9–16 undergoes functional testing to meet specifications for library generation performance.

We recommend the use of control samples when beginning experiments and/or using a new source of samples. For RNA-based experiments, such as RNA-Seq, we recommend using the MicroArray Quality Control (MAQC) reference samples A and B. For DNA-based experiments, such as WGS and exome sequencing, we recommend using a commercial source of genomic DNA.

I. Introduction

This product contains components with multiple storage temperatures.

D. Storage and Stability

Ovation SP+ Ultralow Library Systems reagents are shipped in two boxes. Box 1 is shipped on dry ice and should be stored at –20°C on an internal shelf of a freezer without a defrost cycle. Box 2 is shipped at room temperature, but contains components with multiple storage temperature requirements and should be unpacked immediately upon receipt.

- Vials labeled Agencourt® RNAClean® XP Beads (clear cap) should be removed from the top of the Box 2 shipping carton upon delivery and stored at 4°C.
- All other Box 2 components should be stored at room temperature.

The kit has been tested to perform to specifications after as many as four freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months.

E. Material Safety Data Sheet (MSDS)

An MSDS for this product is available on the NuGEN website at www.nugeninc.com/nugen/index.cfm/support/user-guides/

II. Components

A. Reagents Provided

Table 1. Ovation SP+ Ultralow DR Multiplex Systems 1–8 and 9–16 Components and Reagents (Part Nos. 8133 annd 8134)

PART NUMBER	DESCRIPTION	BOX	VIAL CAP	VIAL NUMBER
S01627	End Repair Buffer Mix	1 of 2	Blue	ER1 VER 5
S01510	End Repair Enzyme Mix	1 of 2	Blue	ER2 VER 4
S01626	End Repair Enhancer	1 of 2	Blue	ER3
S01625	End Repair Enhancer Buffer Mix	1 of 2	Blue	ER4
S01662	Ligation Buffer Mix	1 of 2	Yellow	L1 VER 5
8133 S01787 S01788 S01789 S01790 S01791 S01792 S01793 S01794 **8134** S01795 S01796 S01797 S01798 S01799 S01800 S01801 S01802	DR Multiplex Ligation Adaptor Mixes	1 of 2	Yellow	**8133** L2V13DR-BC1 L2V13DR-BC2 L2V13DR-BC3 L2V13DR-BC4 L2V13DR-BC5 L2V13DR-BC6 L2V13DR-BC7 L2V13DR-BC8 **8134** L2V13DR-BC9 L2V13DR-BC10 L2V13DR-BC11 L2V13DR-BC12 L2V13DR-BC13 L2V13DR-BC14 L2V13DR-BC15 L2V13DR-BC16
S01467	Ligation Enzyme Mix	1 of 2	Yellow	L3 VER 4
S01804	Amplification Buffer Mix	1 of 2	Red	P1 VER 5
S01803	Amplification Primer Mix	1 of 2	Red	P2 VER 11
S01805	Amplification Enzyme Mix	1 of 2	Red	P3 VER 4
S01668	DMSO	1 of 2	Brown	P4
S01001	Nuclease-free Water	1 of 2	Green	D1

II. Components

Ovation SP+ Ultralow DR Multiplex Systems 1–8 and 9–16 Components and Reagents (Part Nos. 8133 annd 8134) *continued*				
PART NUMBER	**DESCRIPTION**	**BOX**	**VIAL CAP**	**VIAL NUMBER**
P01208	Mondrian SP+ Cartridges (x4)	2 of 2	N/A	N/A
S01719	SP+ Cartridge Filler Fluid (x4)	2 of 2	N/A	N/A
P01185	Filler Fluid Vial (x4)	2 of 2	N/A	N/A
S01556	Sample Concentration Solution	2 of 2	Clear	N/A
S01588	Bead Binding Solution (x2)	2 of 2	Clear	N/A
S01589	Bead Wash Solution (x2)	2 of 2	Clear	N/A
S01590	Elution Buffer	2 of 2	Clear	N/A
P01210	Ovation SP+ Ultralow Cartridge Loading Guide (x4)	2 of 2	N/A	N/A
S01698	Agencourt RNAClean XP Beads	Shipped separately	Clear	N/A

II. Components

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Mondrian SP+ Workstation (NuGEN Part No. 8100)
  - Covaris™ S-series Sonication System to fragment input DNA
  - Agilent 2100 Bioanalyzer or materials and equipment for electrophoretic analysis of nucleic acids
  - Microcentrifuge for individual 0.5 mL and 0.2 mL tubes
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 200–1000 µL pipette
  - Vortexer
  - Qubit® 2.0 Fluorometer and dsDNA HS Assay Kit (Life Technologies) or appropriate fluorometer and accessories for quantitation of fragmented DNA and amplified libraries.
- **Reagents**
  - Agilent High Sensitivity DNA Kit (Agilent, Cat. #5067-4626)
  - Isopropyl alcohol
  - Low EDTA TE buffer, pH 8.0 (Affymetrix, Cat. #75793)
- **Supplies and Labware**
  - Nuclease-free pipette tips
  - 0.5 mL and 0.2 mL DNase-free low-bind microcentrifuge tubes (e.g., Sorenson BioScience, Inc. SafeSeal™ Microcentrifuge Tubes with Low Binding Polymer Technology, 0.65 mL, Cat. #11300; Eppendorf DNA LoBind consumables, PCR clean^2 Safe-Lock Tube, 0.5 mL, U.S.A. Cat. #022431005, international Cat. #0030 108.035)
  - Disposable gloves
  - Lint-free wipes such as: Kimwipes™ or Berkshire Super PolX® 1200 Wipers (VWR, Cat. #21914)
  - Canned air
  - Ice bucket
  - Cleaning solutions such as DNA-OFF™ (MP Biomedicals, Cat. #QD0500)

To Order:

- Affymetrix, www.affymetrix.com
- Agilent, www.agilent.com
- Covaris, www.covarisinc.com
- Eppendorf, www.eppendorf.com
- Life Technologies, www.lifetechnologies.com
- MP Biomedicals, www.mpbio.com
- Sorenson BioScience, www.sorbio.com
- VWR, www.vwr.com

III. Planning the Experiment

A. Input DNA Requirements

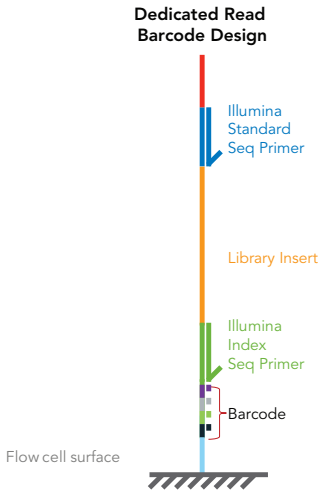
The Ovation SP+ Ultralow Library Systems are designed to work with 10 to 100 ng of fragmented genomic dsDNA or ds-cDNA. The recommended amount of dsDNA template for the Ovation SP+ Ultralow assay depends the type of experiment and the complexity of the template genome. Experiments involving more complex genomes require more dsDNA as template. We recommend using at least 10 ng of input dsDNA for whole genome sequencing (WGS) of complex eukaryotic genomes.

DNA samples must be free of contaminating proteins, RNA, organic solvents (including phenol and ethanol) and salts. We recommend using a commercially available system for DNA/cDNA isolation. The A260:A280 ratio for DNA samples should be in excess of 1.8. Use of DNA samples with lower ratios may result in low amplification yield. DNA samples of excessively low quality or samples that are incorrectly quantitated may yield poor results.

B. Using Ovation SP+ Ultralow Library Systems on Illumina NGS Systems

The Ovation SP+ Ultralow DR Multiplex Systems 1–8 and 9–16 use a 'Dedicated Read' (DR) or second sequencing primer approach for multiplex sequencing. Figure 2 depicts the DR multiplex barcode strategy.

Figure 2. Dedicated read multiplexing strategy used by the Ovation SP+ Ultralow Library Systems.



The Ovation SP+ Ultralow Library Systems use the same approach to multiplexing used in the standard Illumina method. These libraries should be sequenced using the Illumina protocol for multiplex sequencing. The DR barcode sequences are found in

Appendix A of this user guide and must be entered into the Illumina software prior to the analysis.

C. Amplified Library Storage

Amplified libraries may be stored at –20°C.

IV. Protocol

A. Overview

The library preparation process used in the Ovation SP+ Ultralow Library Systems is performed on the Mondrian SP+ Workstation and takes 6–7 hours to complete. After collection of the droplets from the Mondrian SP+ Cartridge, we recommend performing library quantitation and qualification prior to sequencing.

B. Protocol Notes

- The system is designed and intended for processing eight samples at a time. Do not attempt to prepare smaller volume master mixes or process fewer than eight samples using the Ovation SP+ Ultralow Library Systems.
- We recommend the routine use of a positive control DNA. Especially the first time you set up a reaction, using a positive control DNA will allow you to establish a baseline of performance and provide the opportunity to become familiar with the protocol.
- Use the water provided with the kit (green: D1) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Thaw components used in each step and immediately place them on ice.
- Always keep thawed reagents on ice unless otherwise instructed.
- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve it completely prior to use. Buffers, adaptors and primers may be thawed at room temperature followed by brief vortexing.
- Do not warm any enzyme mixes. A gentle mix and quick spin down of enzyme mixes is recommended.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer.
- When instructed to pipet mix, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Components and reagents from other NuGEN® kits should not be used with the Ovation SP+ Ultralow Library Systems.

C. DNA Fragmentation

Use a Covaris S-series System to fragment your source double-stranded gDNA or cDNA to the desired length following the manufacturer's recommendations. The fragmented DNA must be concentrated or purified prior to loading on the Mondrian SP+ Cartridge. We have evaluated only Covaris fragmented DNA during the development of the Ovation SP+ Ultralow Library Systems. Other mechanical means of fragmentation, such as sonication, may also be suitable.

D. DNA Concentration after Fragmentation

The fragmented DNA may require concentration in order to achieve the desired input. This can be done using the Agencourt RNAClean XP bead-based purification protocol detailed below, provided for your convenience. Alternatively, you may choose a column-based purification system that allows small volume elution, such as the MinElute® Reaction Cleanup Kit (QIAGEN®, Cat. #28204).

The purification beads should be removed from 4°C and left at bench top to reach room temperature well before the start of purification.

1. Remove the Agencourt RNAClean XP purification beads from 4°C and place on bench top. Ensure the beads have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting or briefly vortexing the tube. Ensure the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.
3. At room temperature, add 1.8 volumes of the bead suspension to each sample. For example, if the fragmented DNA is in a 50 µL volume, add 90 µL of the bead suspension.
4. Mix thoroughly by pipetting 10 times. It may be helpful to use a multichannel pipettor to ensure the incubation times are uniform.
5. Incubate at room temperature for 10 minutes.
6. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
7. Carefully remove the binding buffer and discard it. Leave ~15 µL behind to minimize bead loss at this step.

   Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the amount of DNA carried into ligation, so ensure beads are not removed with the binding buffer or the wash.
8. With the plate still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
9. Remove the 70% ethanol wash using a pipette.
10. Repeat the 70% ethanol wash two more times, for a total of three washes.

    Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.
11. Air dry the beads on the magnet for a minimum of 10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
12. Remove the tubes from the magnet.
13. Add 28 µL 1X TE buffer (low EDTA) to the dried beads. Mix thoroughly to ensure all the beads are resuspended.

14. Transfer the tubes to the magnet and let stand for 2 minutes.
15. Carefully remove 25 µL of the eluate, ensuring as few beads as possible are carried over, and transfer to a fresh set of tubes. When pipetting any portion of this eluted library downstream, be sure to let stand briefly on a magnet to minimize bead carryover.
16. At this point, we recommend quantitating your samples using a Qubit or other high sensitivity dsDNA quantitation system.

E. Cartridge Quality Control Check

The Mondrian SP+ Cartridge QC protocol confirms the basic functionality of the Mondrian SP+ Cartridge prior to use. We recommend running this protocol with each cartridge prior to preparing or adding samples and reagents.

1. We recommend cleaning the contact pins on the deck of the workstation prior to running the Mondrian SP+ Cartridge QC Protocol. The cleaning procedure is detailed in Appendix C.
2. On the bench top, fill the cartridge with Filler Fluid via the Filler Fluid port, according to the instructions in Appendix B.
3. Carefully transport the cartridge to the workstation and insert it into the deck.
4. Pull the cartridge lever of the Mondrian SP+ Workstation forward to the locked position.
5. Place the Cartridge Loading Guide on the cartridge or use the guide as a reference to identify the correct port for loading Elution Buffer.
6. Pipette 50 µL of Elution Buffer into port E5 of the Mondrian SP+ Cartridge. Insert the pipette tip into the port all the way to the bottom of the cartridge. When the tip contacts the bottom, withdraw the pipette tip slightly to allow space for dispensing. Slowly depress the plunger to dispense the reagent, but do not depress the plunger completely (blow out) as this could introduce bubbles into the cartridge. Slowly withdraw the pipette tip from the port, performing a final blowout while the tip is within the upper cylinder of the port.

   Note: Do NOT add any samples or other reagents to the cartridge at this time. Ensure that only Elution Buffer has been loaded.
7. Close the lid.
8. Select 'Run' on the touch screen menu, choose the Mondrian SP+ Cartridge QC protocol from the list of protocols and then select 'Next' to proceed to the Protocol Information screen.
9. Select 'Next' to proceed to the Run Information screen.

10. **Optional:** Enter run details on the Run Information screen. We recommend recording the serial number of the cartridge on this screen. The cartridge serial number can be found on the front of each cartridge for easy reference.
11. Select 'Next' and then select 'Start Run'.

The Mondrian SP+ Cartridge QC protocol will take about 15 minutes to complete. During this test, Elution Buffer droplets will be dispensed from the E5 port and transported around the cartridge prior to being returned to port E5. The purpose of this test is to confirm the basic performance of the cartridge.

At the end of the protocol, the instrument will display the Run Complete screen and one of the following messages:

MESSAGE	MEANING	NEXT STEP
Mondrian™ SP+ Cartridge passed. Continue to intended protocol.	No errors were detected. Droplet transport was normal. Cartridge is ready to run a protocol	Press "OK" on the Run Complete screen to return to the main menu. Proceed to the intended protocol.
Mondrian™ SP+ Cartridge failed. Remove cartridge from instrument deck and set aside prior to contacting NuGEN Technical Support.	A problem was detected with droplet transport within the cartridge.	Press 'OK' on the Run Complete screen to return to the main menu. Carefully remove the cartridge from the deck of the workstation, setting aside the cartridge for possible return to NuGEN. Begin with a new cartridge and contact NuGEN Technical Support to request a replacement for the failed cartridge.

F. Protocol for the Ovation SP+ Ultralow Library Systems on the Mondrian SP+ Cartridge

1. Sample Solution Preparation

Preparation of the sample solution is done on a per sample basis and not as a master mix. You must prepare and process eight samples on each cartridge. We recommend starting sample preparation and reagent preparation once the cartridge has successfully completed QC.

Table 2. Sample Solution Preparation (volumes listed are for a single sample)

COMPONENT	VOLUME
10 to 100 ng sheared dsDNA in water or low EDTA TE	23.5 µL
Agencourt RNAClean XP beads	4.0 µL
Sample Concentration Solution	27.5 µL
Total volume	**55 µL**

Ensure Agencourt RNAClean XP beads are at room temperature and completely resuspended prior to use. Mix each sample solution well and incubate at room temperature (approximately 23°C) for 10 minutes. The above recipe is for a single sample. Prepare this sample solution for each sample to be processed. Mix the samples once again before loading onto the cartridge.

Note: We recommend using a consistent amount of input material across all lanes of a cartridge. Consistent input amounts reduce the chances of over- or under-amplification across the cartridge as the same protocol and PCR conditions must be used for the entire cartridge.

2. Ovation SP+ Ultralow Reagent Master Mix Preparation

Prepare Bead Binding Solution, Bead Wash Solution and Elution Buffer

1. Remove the Bead Binding Solution, Bead Wash Solution and Elution Buffer from the room temperature reagent box.
2. Vortex to mix and spin down briefly. Leave tubes at room temperature while preparing the additional master mixes below.

Prepare DR Multiplex Barcode Adaptors

1. Thaw DR Multiplex Ligation Adaptor Mixes (L2V13DR-BC1–8 or L2V13DR-BC9–16) at room temperature.
2. Vortex well to mix and spin down briefly. Leave tubes at room temperature while preparing the additional master mixes below.

The ligation buffer is very viscous and must be pipetted slowly to ensure complete aspiration and transfer.

Prepare Ligation Master Mix

1. Thaw Ligation Buffer Mix (L1 VER 5) at room temperature and vortex to mix well. Spin and place the Ligation Enzyme Mix (L3 VER 4) on ice.
2. Prepare master mix in a low-binding 0.5 mL microcentrifuge tube or 0.2 mL PCR tube according to the volumes shown in Table 3. Label the tube D5.
3. Mix well by carefully pipetting, avoiding the introduction of bubbles. Briefly spin down to bring the master mix to the bottom of the tube.

Table 3. Ligation Master Mix (label tube D5)

COMPONENT	VIAL CAP	VOLUME
Ligation Buffer Mix (L1 VER 5)	Yellow	7.0 µL
Ligation Enzyme Mix (L3 VER 4)	Yellow	3.0 µL
Total volume		**10.0 µL**

Prepare End Repair Master Mix

1. Thaw End Repair Buffer Mix (ER1 VER 5) at room temperature, vortex to mix well and spin down briefly. Briefly spin down the End Repair Enzyme Mix (ER2 VER 4) and place on ice.
2. Prepare master mix in a low-binding 0.5 mL microcentrifuge tube or 0.2 mL PCR tube according to the volumes shown in Table 4. Label the tube D6.
3. Mix well by carefully pipetting, avoiding the introduction of bubbles. Briefly spin down to bring the master mix to the bottom of the tube.

Table 4. End Repair Master Mix (label tube D6)

COMPONENT	VIAL CAP	VOLUME
End Repair Buffer Mix (ER1 VER 5)	Blue	9.0 µL
End Repair Enzyme Mix (ER2 VER 4)	Blue	1.0 µL
Total volume		**10.0 µL**

Prepare End Repair Enhancer Master Mix

1. Thaw End Repair Enhancer Buffer Mix (ER4) at room temperature, vortex to mix well and spin down briefly. Spin and place the End Repair Enhancer (ER3) on ice.
2. Prepare master mix in a low-binding 0.5 mL microcentrifuge tube or 0.2 mL PCR tube according to the volumes shown in Table 5. Label the tube D7.
3. Mix well by carefully pipetting, avoiding the introduction of bubbles. Briefly spin down to bring the master mix to the bottom of the tube.

Table 5. End Repair Enhancer Master Mix (label tube D7)

COMPONENT	VIAL CAP	VOLUME
End Repair Enhancer Buffer Mix (ER4)	Blue	7.0 µL
End Repair Enhancer (ER3)	Blue	3.0 µL
Total volume		**10.0 µL**

Prepare Library Amplification Master Mix

! *DMSO will not completely thaw on ice. Thaw at room temperature and vortex to mix well.*

1. Thaw Amplification Buffer Mix (P1 VER 5), Amplification Primer Mix (P2 VER 11), and DMSO (P4) at room temperature and vortex to mix well. Spin and place the Amplification Enzyme Mix (P3 VER 4) on ice.
2. Prepare Library Amplification Master Mix in a low-binding 0.5 mL microcentrifuge tube or a 0.2 mL PCR tube according to the volumes shown in Table 6. Label the tube E2.
3. Mix well by carefully pipetting, avoiding the introduction of bubbles. Briefly spin down to bring the master mix to the bottom of the tube.
4. Keep the prepared Library Amplification Master Mix on ice.

Table 6. Library Amplification Master Mix (label tube E2)

COMPONENT	VIAL CAP	VOLUME
Amplification Buffer Mix (P1 ver 5)	Red	14.2 µL
Amplification Primer Mix (P2 ver 11)	Red	6.7 µL
DMSO (P4)	Brown	1.6 µL
Amplification Enzyme Mix (P3 ver 4)	Red	2.5 µL
Total volume		**25.0 µL**

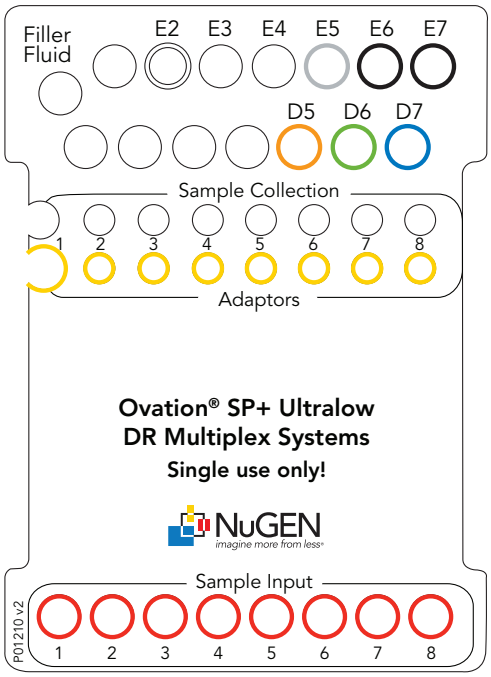
3. Mondrian SP+ Cartridge Loading Instructions

Loading Reagents and Samples in the Mondrian SP+ Cartridge

If you have confirmed the integrity of the Mondrian SP+ Cartridge through the Mondrian SP+ Cartridge QC Protocol, it is not necessary to add any additional Filler Fluid to the cartridge prior to loading samples and reagents. If the Mondrian SP+ Cartridge QC Protocol has not been performed, you must add Filler Fluid to the cartridge prior to loading samples and reagents. Place the cartridge on a level surface and fill the cartridge with filler fluid according to the Mondrian SP+ Cartridge Handling instructions in Appendix B.

IV. Protocol

Figure 3. Cartridge loading guide for the Ovation SP+ Ultralow Library System protocol.



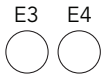
Follow the instructions below to load reagents into their appropriate cartridge ports.

- Use a 10 or 20 µL pipette to add the adaptors and all reagent master mixes, except for the Library Amplification Master Mix.
- Use a 100 or 200 µL pipette for adding the samples, Bead Binding Solution, Elution Buffer, Library Amplification Master Mix and Bead Wash Solution.
- Load the ports in a steady manner to avoid overflow of the Filler Fluid.
- When adding sample or reagent, lower the pipette tip to the bottom of the port. Do not press the tip into the bottom of the cartridge. If the tip contacts the bottom of the cartridge withdraw the pipette tip slightly upwards. Slowly depress the plunger to the first stopping point to dispense the reagent completely from the pipette tip. As you raise the pipette tip from the port, you should complete a gentle blow-out by fully depressing the pipette plunger just prior to exiting the filler fluid to ensure all the reagent is dispensed.

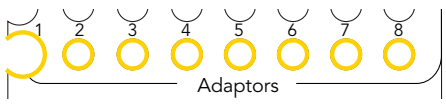
Note: Using a desktop lamp to illuminate your work area may facilitate sample and reagent loading. We recommend loading the reagents and samples with the cartridge on the deck of the Mondrian SP+ Workstation.

IV. Protocol

Important: It is critical to use a 10 or 20 µL pipette for adding any volumes of ≤10 µL. Do not use 2 µL pipettes to add reagents to the cartridge as they will not generate sufficient force to expel the reagents from the pipette tip into the Filler Fluid-containing cartridge.

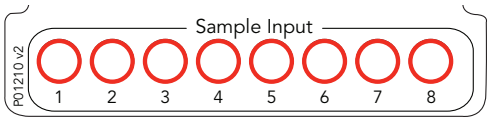
1. Load 25 µL Library Amplification Master Mix into port E2 (white rim).	
2. Load 50 µL Bead Binding Solution into ports E3 and E4 (no color).	
3. If cartridge QC has not been completed, load 50 µL Elution Buffer into port E5 (grey rim). If cartridge QC has been completed, skip this step and proceed to step 4.	
4. Load 50 µL Bead Wash Solution into ports E6 and E7 (black rim).	
5. Load 8 µL Ligation Master Mix into port D5 (orange rim).	
6. Load 8 µL End Repair Master Mix into port D6 (green rim).	
7. Load 8 µL End Repair Enhancer Master Mix into port D7 (blue rim).	

8. Load 1.5 µL of each DR Multiplex Ligation Adaptor Mix (L2V13DR-BC1–8 or L2V13DR-BC9–16) into the appropriate port (A1 through A8 , yellow rims) matching the sample to be barcoded, ensuring that the adaptors are carefully dispensed at the bottom of the port.



Note: If the adaptor droplet appears to be floating above the surface of the bottom plate of the cartridge, use a clean pipette tip to gently push the droplet down to the surface.

9. Load 50 µL of sample mix into S1–S8 (red rims). Ensure that the 10-minute incubation step described above is performed prior to loading onto the cartridge. Mix the samples once more before loading.



10. If the cartridge is not already on the Mondrian SP+ Workstation deck, carefully transport the cartridge to the Mondrian SP+ Workstation, insert the cartridge into the deck, pull the locking lever and close the lid.

Mondrian SP+ Workstation Initialization Instructions

1. If not already ON, locate the workstation ON/OFF switch at the back of the workstation and turn it to ON.
2. Press the 'On' button on the front of the workstation.
3. Select 'Run' from the Main Menu touch screen, then select the Ovation SP+ Ultralow protocol with the desired number of cycles (7, 9, 11, 13, or 15) from the Protocol Selection menu. Only one protocol may be run per cartridge. As a general rule, the greater the amount of input DNA, the fewer cycles of amplification should be performed. For inputs of 50 to 100 ng, choose 7 or 9 cycles. For inputs of 10 to 50 ng, choose 11 or more cycles. Note that these are only recommendations. The actual number of amplification cycles should be determined empirically by the user.
4. After you have selected a protocol, press 'Next'. Follow the instructions on the screen to begin the run.
5. When the run is complete (approximately 7 hours) continue to Library Collection.

Library Collection from the Mondrian SP+ Cartridge

Note: A short instructional video on 'Mondrian Sample Collection' is available on the Mondrian Support page at www.nugeninc.com/mondriansupport.

1. Place eight low-binding 0.2 mL or 0.5 mL microcentrifuge tubes in a rack. Add 15 µL of Nuclease-free Water (green: D1) to each tube.
2. Use a 100 or 200 µL pipette set to 20 µL. Depress the plunger on the pipette and insert the tip all the way to the bottom of the sample collection port, perpendicular to the cartridge to make a seal between the cartridge and the pipet tip.

3. Maintaining the seal formed between the pipette tip and the bottom of the cartridge, release the plunger. Immediately lift the pipette slightly off the bottom of the cartridge to release the seal, rapidly drawing Filler Fluid and the sample droplet into the pipette tip.
4. Examine the pipette tip to ensure that the appropriately sized droplet is suspended in the Filler Fluid.
5. Dispense the collected fluid including the small sample droplet directly into the 15 µL of water in the collection tube. Pipette up and down a few times in the aqueous phase to ensure that all the sample transfers into the water.
6. Discard the tip and repeat steps 2–5 to ensure complete collection of the entire sample. This is important because occasionally a portion of the sample droplet is left behind in the sample collection port.
7. Continue to the next sample collection port and repeat this process until all eight libraries have been collected and placed in separate tubes.
8. Remove the cartridge from the workstation and dispose of as appropriate in laboratory waste.
9. Cap the library droplet-containing PCR tubes and vortex briefly to mix. Spin down briefly to bring the aqueous phase to the bottom of the tube. The Filler Fluid oil should remain as a separate layer on top of the aqueous phase.
10. Use a pipette to either remove and transfer the lower aqueous phase to a new tube or to remove and discard the Filler Fluid oil layer.
11. The aqueous phase contains the purified amplified library. Store the library at –20°C or proceed immediately to library quantitation.

V. Quantitative and Qualitative Assessment of the Purified Amplified Libraries

A. Overview

The Quantitative and Qualitative Assessment is used to confirm the average size of the library inserts and calculate library concentration. This information is required prior to loading the cluster generation workstation.

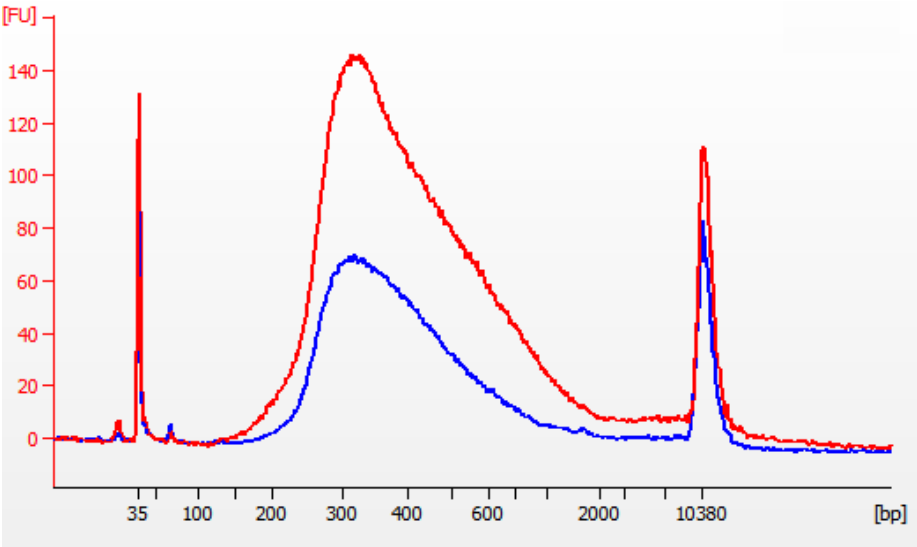
B. Recommendations for Library Assessment

1. Run a 1 µL aliquot of the library on the Bioanalyzer High Sensitivity (HS) DNA Chip. The typical distribution of libraries with 200 bp inserts is shown in Figure 4.

   Note: Libraries appear to be closer to 300 bp due to the additional length of ligated adaptors.

The distribution pattern of the amplified library inserts depends upon the initial fragment size used to construct the library. Following the Ovation SP+ Ultralow protocol effectively eliminates adaptor dimer formation. Any deviation from the protocol may result in dimer formation, which will appear as low molecular weight spikes of approximately 120 bp on the Bioanalyzer trace.

Figure 4. Ovation SP+ Ultralow Library Size Distribution. Size distribution of libraries generated from 10 ng of gDNA (blue trace) or 50 ng of gDNA (red trace) run on a High Sensitivity DNA Chip.



2. To quantitate the libraries, we recommend using a fluorescence-based double-stranded DNA quantification system, such as the Qubit Fluorometer (Life Technologies), or a qPCR-based method such as the KAPA Library Quantification System (KAPA Biosystems). Yields will vary, depending on input and choice of PCR enrichment cycle number. Typical yields are 50–100 ng.

VI. Technical Support

For help with any of our products, please contact NuGEN Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only). You may also send faxes to 888.296.6544 (toll-free) or email techserv@nugeninc.com.

In Europe contact NuGEN at +31(0)135780215 (Phone) or +31(0)135780216 (Fax) or email europe@nugeninc.com.

In all other locations, contact your NuGEN distributor for technical support.

VII. Appendix

A. Sequences of the Barcodes in the Multiplexed Reactions

Barcode sequences and multiplex guidelines for adaptors used in Ovation SP+ Ultralow DR Multiplex Systems 1–8 and 9–16 can be found in Table 7 and Table 8, respectively. These 6-nucleotide barcode adaptor sequences must be input into the Illumina Sequencing System prior to parsing of the data.

You may combine anywhere from 2–16 barcoded libraries to allow for a range of multiplex sequencing. However, the barcodes were carefully chosen for their ability to parse properly and for color balancing and therefore have strict pairing requirements when performing 2-plex multiplexing. Users wishing to perform greater than a 2-plex multiplexing must choose a Duplex Set (as defined in Tables 10 and 11), combined with any of the remaining barcoded libraries.

All barcode sequences are separated by an edit distance of three. For further details on the barcode design strategy, please refer to Faircloth BC, Glenn TC (2012) Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. PLoS ONE 7(8): e42543. doi:10.1371/journal.pone.0042543.

Table 7. Barcode sequences for dedicated read (DR) adaptors used in Ovation SP+ Ultralow DR Multiplex System 1–8 (Part No. 8133).

LIGATION ADAPTOR MIX	6 NT BARCODE SEQUENCE AS READ BY THE SEQUENCER	BARCODE PAIRING (2-PLEX)	BARCODE PAIRING (> 2-PLEX)
L2V13DR-BC1	AACCAG	Duplex Set 1	One of the duplex sets from the column to the left must be used in combination with any of the other remaining six individual barcodes.
L2V13DR-BC2	TGGTGA		
L2V13DR-BC3	AGTGAG	Duplex Set 2	
L2V13DR-BC4	GCACTA		
L2V13DR-BC5	ACCTCA	Duplex Set 3	
L2V13DR-BC6	GTGCTT		
L2V13DR-BC7	AAGCCT	Duplex Set 4	
L2V13DR-BC8	GTCGTA		

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Table 8. Barcode sequences for dedicated read (DR) adaptors used in Ovation SP+ Ultralow DR Multiplex System 9–16 (Part No. 8134).

LIGATION ADAPTOR MIX	6 NT BARCODE SEQUENCE AS READ BY THE SEQUENCER	BARCODE PAIRING (2-PLEX)	BARCODE PAIRING (> 2-PLEX)
L2V13DR-BC9	AAGAGG	Duplex Set 1	One of the duplex sets from the column to the left must be used in combination with any of the other remaining six individual barcodes.
L2V13DR-BC10	GGAGAA		
L2V13DR-BC11	AGCATG	Duplex Set 2	
L2V13DR-BC12	GAGTCA		
L2V13DR-BC13	CGTAGA	Duplex Set 3	
L2V13DR-BC14	TCAGAG		
L2V13DR-BC15	CACAGT	Duplex Set 4	
L2V13DR-BC16	TTGGCA		

B. Mondrian SP+ Cartridge Handling

Background

The Mondrian™ SP+ Cartridge is used with the Mondrian SP+ System and offers a convenient 8-sample batch size, simple reagent loading and easy sample recovery. Reagents are contained in discrete droplets encased within filler fluid, isolating the reactions from the lab environment and carryover contamination.

Construction

When prepared for use, cartridges consist of an oil layer (Filler Fluid) sandwiched between a PCB substrate and a clear top plate. The PCB substrate is patterned with insulated electrodes. By changing the relative voltages of the patterned electrodes, aqueous droplets are manipulated to perform complex assays. Droplets are dispensed from loading ports, transported to various locations on the cartridge, mixed, incubated and collected using only software control. Figure 5 shows a cutaway side view schematic of a cartridge.

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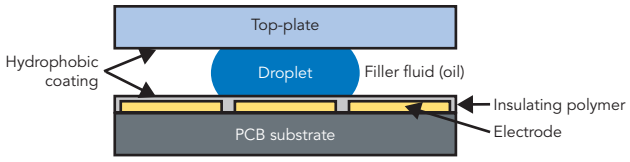
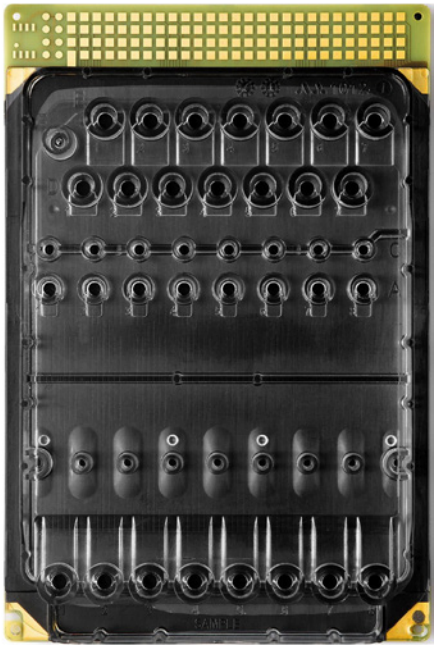
Figure 5. Cartridge cutaway side view.



Cartridges

Cartridges are one-time-use only. Cartridge re-use is not supported, even if a run is canceled prior to completion.

Figure 6. Top view of a Mondrian SP+ Cartridge. Sample ports along the bottom edge are labeled S1–8. Other reagent port rows are labeled A–E. Note that there is no row B. There are eight ports in rows A and C, and seven in rows D and E. The port labeled "F" is sized for a standard Luer-lock connector, through which the Filler Fluid is added to the cartridge. The cartridge electrodes are visible as rows of small gold squares at the top of the cartridge.


F: Filler Fluid port
E: Reagents and master mixes
D: Reagents and master mixes
C: Collection of final, purified libraries
A: Adaptor ports
Bubble trap ports: nothing is added or removed via these ports
Sample input ports

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Important: Take care when removing the cartridge from the packaging. Handle the cartridge by the sides and the non-electrode end. Do not handle the cartridge by the electrodes.

Filling the Cartridge

SP+ Cartridges must be filled with the provided Filler Fluid prior to loading with reagents. This process should be carried out on the bench top.

1. Use scissors to cut open the Mondrian SP+ Cartridge pouch. Remove the Mondrian SP+ Cartridge.

   Note: Do **not** attempt to tear open the pouch as this may damage the SP+ Cartridge.

2. Lay the cartridge on a level bench top and place the appropriate SP+ Library Systems Cartridge Loading Guide on the cartridge.
3. Remove the empty Filler Fluid tube and the SP+ Filler Fluid bottle from the NuGEN SP+ Library System kit.
4. Carefully grasp the tab on the red aluminium seal of the SP+ Filler Fluid bottle and pull up and back to remove the seal.
5. Remove the top and bottom caps from the empty Filler Fluid tube.
6. Attach the empty Filler Fluid tube to the SP+ Cartridge by inserting the tip of the Filler Fluid tube into the port marked "Filler Fluid" on the cartridge while gently twisting the tube in a clockwise direction to secure the Filler Fluid tube on the cartridge.
7. Remove the rubber stopper from the bottle of SP+ Filler Fluid.
8. Slowly pour the SP+ Filler Fluid into the Filler Fluid tube. Be careful not to pour too quickly as this may introduce bubbles into the fluid. Air bubbles entering the cartridge may interfere with droplet movement. During the cartridge filling process, you will begin to see the Filler Fluid rising in the sample and reagent ports. You should see Filler Fluid in all sample and reagent ports by the end of the filling process.
9. Once the entire volume of Filler Fluid has been delivered, carefully remove the Filler Fluid tube from the SP+ Cartridge.

! *The SP+ Filler Fluid has been degassed and packaged in a sealed, single-use vial. Do not open the vial until ready for use.*

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Cartridge Insertion

1. Raise the lid of the Mondrian SP+ Workstation to reveal the cartridge deck.

2. With the cartridge lever in the up position, as shown above, hold the cartridge level by the sides with the ports facing up and the gold colored electrodes pointing towards the workstation. Carefully insert the cartridge into the cartridge deck flush with the surface of the deck, allowing the side rails to guide it into place.

3. If you encounter any resistance to the cartridge moving into the deck, inspect for foreign objects that may be impeding the cartridge and remove them. If the cartridge appears to be catching on the edge of the heater bars or Peltier cooling plate, you may gently press down on them to allow the cartridge to move forward. The heater bars and cooling unit are designed to be slightly flexible in order to engage the cartridge. However, do not press too hard on the heater bars or Peltier unit as they may become misaligned.

4. Confirm that the cartridge is fully inserted into the deck. When the cartridge is properly inserted, the electrodes are no longer visible and the three guide arrows etched into the front of the deck should be flush with the edge of the cartridge.

5. Pull the locking lever forward as illustrated below to engage the control electronics and lock the cartridge into place.

   Note: Once a run has started, do not move the locking lever as this will result in an unrecoverable interruption of the running protocol. The Mondrian SP+ Workstation will not run with the lever in the up position.

C. Cleaning the Workstation Contact Pins

The interface pins at the workstation/cartridge interface may become dirty and cause performance issues. If this happens, you will need to clean the pins.

Materials

- Lint-free wipes such as Kimwipes or Super PolX 1200 Wipers. Do not use cotton or any material that may leave particles behind
- Isopropyl alcohol
- Canned air

Procedure

1. Turn the workstation off and unplug from the power source.
2. Soak the wipe in isopropyl alcohol.
3. Gently insert a folded wipe into the interface pin area at the back of the cartridge deck and rub all pins with the wipe.
4. Wait 2 minutes for the isopropyl alcohol to evaporate.

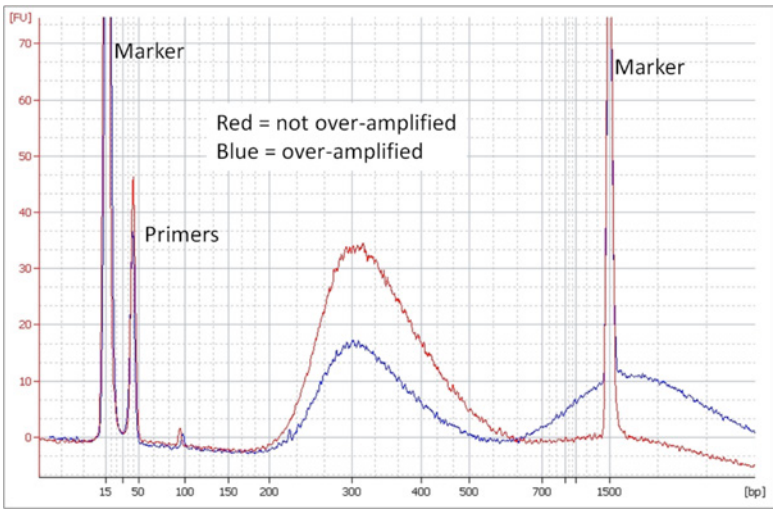
5. Blow the area dry with canned air.
6. Plug the workstation back in and turn it on.

Note: We recommend cleaning the pins once a week, after any prolonged storage of the workstation, after a Filler Fluid spill, or if the Mondrian SP+ Cartridge QC protocol returns a 'Mondrian SP+ Cartridge Failed' or a 'Mondrian SP+ Cartridge Status Undetermined' message.

D. PCR Amplification Artifacts

In some instances, PCR amplification may create artifacts in the downstream library size analysis. These artifacts appear as high molecular weight species during Bioanalyzer or gel analysis (Figure 7). This phenomenon is due to the amplification of diverse library molecules that have the same adaptor sequences at their termini. As the concentration of library molecules increases during PCR, the adaptor ends begin to compete with the PCR primers for hybridization, resulting in partially hybridized species. Although this may impact PCR efficiency, it does not impact library quality for subsequent sequencing, nor does it affect quantitation by qPCR.

Figure 7. Fragment distribution on Bioanalyzer Chip 1000 when PCR amplification artifacts are present.



If desired, performing a single round of PCR in the presence of excess primer will resolve the material to a single peak of the correct library size.

When quantifying libraries that may have been subject to PCR amplification artifacts, use the lower molecular weight peak to estimate library size and qPCR to determine concentration.

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E. Frequently Asked Questions (FAQs)

Q1. What kind of sequencing primers can I use with your library?
The Ovation SP+ Ultralow Library Systems are designed for use with the standard Illumina sequencing primers for both single end and paired-end sequencing applications.

Q2. Can the Ovation SP+ Ultralow Library Systems be used with paired-end sequencing?
Yes, they can be used for both single end and paired-end sequencing. Special consideration should be given to the expected insert size in the paired-end assay.

Q3. Is there a lower or an upper size limit that I can use to make my library?
We have successfully constructed libraries using fragments ranging from 200 to 500 bp. It is possible to generate a library using both smaller and larger fragments although a specific upper and lower size limit has not been established.

Q4. How much material should I load into the cBot?
Please follow manufacturer's recommendations for library QC, quantitation, balancing and loading of the amplified library on the cBot.

Q5. Do the Ovation SP+ Ultralow Library Systems work with the Illumina Cluster Station (predecessor of the cBot instrument)?
Yes, the Systems are also compatible with the Illumina Cluster Station.

Q6. I don't have access to a Covaris instrument, can I use alternative fragmentation methods?
We have evaluated only Covaris fragmented DNA during the development of the Ovation SP+ Ultralow Library Systems. Other mechanical means of fragmentation, such as sonication, may also be suitable.

Q7. How does your protocol improve the efficiency of ligation and avoid adaptor dimer formation?
The Ovation SP+ Ultralow Library Systems utilize optimized chemistries to increase the efficiency of blunt-end adaptor ligation and minimize the amount of adaptor dimer in the library.

Q8. Does NuGEN provide reagents for performing the fragmentation step of the protocol?
We recommend using the Covaris instrument, as indicated in the "Materials" section of this user guide. NuGEN does not provide the reagents used in the fragmentation steps.

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Q9. Which NuGEN amplification system kits can be used to produce cDNA for input to the Ovation SP+ Ultralow Library Systems?
The Ovation WGA FFPE System (Part No. 6200), Ovation RNA-Seq System V2 (Part No. 7102), Ovation RNA-Seq FFPE System (Part No. 7150), Ovation 3´-DGE System (Part No. 7200), and Ovation Prokaryotic RNA-Seq System (Part No. 9030) have been specifically designed for NGS applications. The cDNAs produced from these systems are compatible with the Ovation SP+ Ultralow Library Systems.

Q10. Are the Ovation SP+ Ultralow libraries compatible with downstream target capture methods like Agilent's SureSelect?
As a general rule, target capture requires a minimum of 500 ng of amplified purified library. The amount of purified, amplified library generated by the Ovation SP+ Ultralow Library Systems is not enough for target capture from non-multiplexed libraries. However, it may be possible to perform target capture on multiplexed libraries where the amount of multiplexed library exceeds 500 ng. You may continue to use the Ovation SP Ultralow Library Systems (Part No. 8033 and 8034) if you wish to perform target capture on the final amplified library without the need to pool libraries prior to capture. Target capture of Ovation SP Ultralow (or Ovation SP+ Ultralow) libraries requires the use of the Encore Target Capture Module (Part No 0332) in addition to the Agilent target capture product.

Q11. How can gel purification be eliminated from the workflow and still prevent adaptor dimer formation?
The Ovation SP+ Ultralow Library Systems workflow uses bead-based purification and efficient primer design, thus eliminating the need for gel-based purification.

Q12. Can I use the Ovation SP+ Ultralow Library System with the Mondrian SP Workstation?
No. The Ovation SP+ Ultralow Library System kit cannot be used on the Mondrian SP Workstation. This protocol is compatible only with the Mondrian SP+ Workstation. Make sure to select the Ovation SP+ Ultralow protocol from the workstation menu when running this kit on the SP+ Workstation.

Q13. What are the expected yields from the Ovation SP+ Ultralow Library System?
Yields will vary depending on the the quantity of DNA input and the number of cycles used in PCR amplification. Typical yields within the recommended input and cycle parameters are 50–100 ng, as measured by Quibit Fluorometer. For information on PCR enrichment cycles, please see the Mondrian SP+ Workstation Initialization Instructions, in the protocol section of the user guide.

VII. Appendix

F. Update History

This document, the Ovation SP+ Ultralow Library System User Guide (M01327 v4) has been updated from the previous version to address the following topics:

Description	Section	Page(s)
Updated protocol for Cartridge Quality Control Check	IV.E.	12–13
Updated volume of reagents to load in wells D5–D7	IV.F.	19

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